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Methylphenidate Produces Conditioned place preference, and cannabidiol Exposure during Extinction does not Inhibit the Reinstatement of Methylphenidate in the Marmoset Monkeys

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Abstract— *Methylphenidate (MPH) is a central nervous system stimulant used as a pharmacotherapy to treat Attention-Deficit/Hyperactivity Disorder and narcolepsy. Scientists are concerned that MPH use could lead to increase the risk of vulnerability to drug abuse later in life. Little work has been carried out on the addictive potential of MPH in non-human primates (NHP). In the present study we intend to evaluate whether the MPH is able to produce a conditioned response and if the exposure to cannabidiol (CBD) during the extinction trial of the conditioned preference place (CPP) paradigm can inhibit the reinstatement of the response in male marmoset monkeys. Animals received alternating intraperitoneal (i.p.) injections of either MPH (5mg/kg) or saline (SAL) to a daily 15 min conditioning trial during 10 consecutive days in drug- and saline-paired compartments, respectively, of a CPP box. After a place preference test the animals were submitted to daily CBD injection in a 15min extinction trial, until the association between MPH and the MPH-paired compartment was extinguished. Then, 24h after the last extinction trial, animals received a priming dose of MPH (1mg/kg) and were submitted to a 15min retest trial. We found that MPH induced strong and long-lasting reinforcing properties during the conditioning period even after extinction training and reinstatement test. Therefore, MPH induced a CPP response in a NHP model and CBD administration could not inhibit the reinstatement of the MPH-induced CPP response.*

Keywords—***Methylphenidate, Cannabidiol, Extinction, Reinstatement, Conditioned place preference, Non-human primates.***

I. INTRODUCTION

While the etiology of attention deficit/hyperactivity disorder is still actively being explored, there is good evidence to suggest that stimulant medications can reduce some of the most common symptoms (1). MPH is, like amphetamine and cocaine, a central nervous system stimulant drug, mainly used as medicine for the treatment of attention deficit/hyperactivity disorder; high repeated exposure to MPH can cause adverse effects in behavior such as increased risk of substance abuse, tolerance or behavioral sensitization by action on the dopaminergic system to elicit a reward response in to MPH (2,3). Also, there is evidence that long-term effects of MPH exposure on the behavior could lead to long-lasting alterations in brain structure, natural reward systems and induce a CPP in both adolescent male and female rats (4). There is general agreement about the observation that endocannabinoid signaling is involved in reward circuitry and memory mechanisms related to extinction and relapse (5). CB1 receptor blockade is effective in reducing cue-induced reinstatement of drug seeking in

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psychostimulants, opiates, nicotine and alcohol addiction. There are no previous studies directly linked methylphenidate uses to biological changes in the endogenous cannabinoid system. The above considerations could suggest that methylphenidate early exposure may alter endocannabinoid or reward (6).

NHP seem to respond more similarly as humans to the effects of MPH treatment. However, only a few studies have investigated the use of MPH as a psychostimulant drug to induce dopamine (DA) release in the common marmoset (7). A study on juvenile rhesus monkey showed cognitive impairments following chronic MPH administration (8). It was also demonstrated that long-term exposure to oral MPH during peri-adolescence has weak effect on physiological or behavioral/cognitive development in NHP (9) and MPH produced no significant changes upon the locomotor activity of young squirrel monkeys (10). There is also only one study that show methylphenidate-induced striatal dopamine release in the common marmoset could be evaluated by [18F] fallypride (7). It has been found that CBD is the major non-psychoactive chemical found in marijuana has an antipsychotic effect and lacks hedonic properties, may help reduce the risk of drug relapse, blocks the reward-facilitating effect of morphine (11) and attenuates cue-induced reinstatement of heroin seeking (12). The CBD appeared to be effective in reducing reinstatement of substance abuse drug and alcohol relapse in rats (13). Nonetheless, the knowledge about the behavioral effects of MPH in marmoset monkeys is still elusive and, to our knowledge, studies looking strictly at CPP behavior in response to pharmacological manipulations of the MPH or CBD in NHP have not been held. The purpose of this study is to evaluate whether MPH can produce a CPP response in male marmoset monkeys (Callithrixpenicillata), and if CBD can inhibit the reinstatement of this response after extinction sessions (1,2).

II. METHODS

Five male adults black-tufted-ear marmosets (*Callithrixpenicillata*) were used, weighing 352±5 (range: 340-365g) at the beginning of the study. Marmosets were pair-housed at the Primate Center of the University of Brasilia in cages (2m x 1m x 2m each) of the same colony room. Marmosets were exposed to natural light, temperature and humidity conditions. All procedures herein were approved by the Animal Ethics Committee of the University of Brasilia and followed NIH/USA guidelines for care and use of laboratory animals.

Testing was conducted in a two-compartment CPP box, suspended 1m from the floor. Each compartment (60cm x 60cm x 35cm) had three walls and the floor made of aluminum, whereas the fourth wall and the top were made of glass (14). Each compartment had different visual and tactile cues. One had a smooth surface and white color, whereas the other had a rough surface and was painted with black and white diagonal stripes. The aluminum wall dividing the CPP box into two compartments consisted of a horizontally-sliding door. If retracted, it gave access to both sides of the apparatus. Each compartment had an independent entry/exit door located on the aluminum side directly opposite the glass wall. Attached to the apparatus, was an aluminum antechamber that encompassed both access doors. The subjects could only access the compartment's sliding doors and enter the respective compartment via this common antechamber, which had a guillotine-type door as its access point.

The CPP box was set-up in a test-room 50m away from the colony facility and subjects were transported between their home-cages and the test-room via a transport-cage (35cm x 20cm x 23cm). This aluminum box prevented them from seeing their surroundings and was attached directly to the guillotine-type door of the CPP box. The apparatus was monitored via a closed-circuit system using two cameras (model C920, Logitech, Brazil): one mounted 1.5m above the arena and other placed 1.5m in front of its glass wall. Both cameras were connected to a same laptop located in an observation-room adjacent to the test-room.

Pills of Methylphenidate hydrochloride (MPH; 5.0mg/kg; Ritalin®, Novartis, Brazil) were macerated and dissolved in phosphate-buffered saline. Cannabidiol (CBD; 30mg/kg; STI Pharm, UK) was dissolved in a 1:19 solution of Tween 80 (Sigma-Aldrich, Brazil) and phosphate-buffered saline, serially. Doses (MPH, CBD) were based on previous studies in primates (7,15). Treatments were given i.p. in a volume of 1ml/kg, 10 min prior to behavioral testing for MPH and 30min for CBD.

Marmosets were submitted to a CPP protocol similar to that used in previous studies from our group (14). Each marmoset initially underwent was initially submitted to a 15 min habituation trial in the CPP box on two consecutive days and no drug was available in neither compartment and the aluminum sliding-wall was kept partially retracted, providing a 30 cm passage between compartments. The marmosets then were submitted to a daily 15 min conditioning trial in the CPP box during 10 consecutive days. On these trials, the common sliding-wall remained shut. On alternate days, each marmoset

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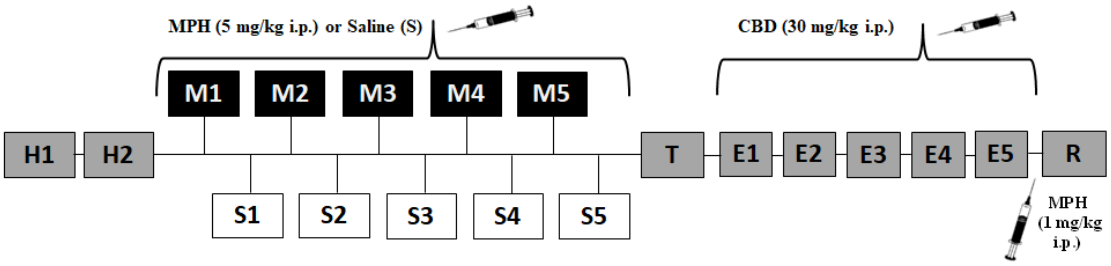
was given access to either the white or striped compartment. Subjects received MPH on odd-numbered trials (i.e., 1, 3, 5, 7 and 9) on the conditioned compartment (CC). On even-numbered trials (i.e., 2, 4, 6, 8 and 10) animals received saline. Animals were arbitrarily conditioned in the white or striped context. Place preference response was determined in a 15 min test trial in the CPP box, 24h after the last conditioning trial. During this trial, each marmoset could access both compartments and no drug was provided, similarly to the habituation trials.

After the test trial, subjects received daily i.p. injections of CBD 30 min prior to entrance into the CPP box for 15 min extinction sessions. These trials were made until the extinction of the place preference response. This extinction was determined when subjects' place preference response was statistically different from the test trial, for two consecutive days. One day after the last extinction trial, one reinstatement trial was made, similarly to the conditions of the test, except that a prime dose (1.0 mg/kg of MPH) was given 10 min before the behavioral test, to evaluate the sensitization and reinstatement (Supplementary Fig. 1).



Supplementary Fig. 1. *Schematic representation including the two initial habituation trials (H1 and H2) that marmosets had free access to the entire CPP box, and then followed by the methylphenidate (MPH, 5 mg/kg; i.p.; M1–M5) and saline-conditioning trials (S1–S5) held on alternative days with access to one of the compartments. Test trial (T) was held after a sequence of ten MPH/saline-conditionings and the extinction period (E1-E5) was held 24 hours after the test phase with a daily injection of cannabidiol (CBD; 30 mg/kg; i.p.) for five consecutive days. During the test and extinction trials, marmosets had free access to both compartments, and no injections were given prior to the habituation and test trials. One day after the last extinction trial, the reinstatement trial (R) was made, and similar to the test trial, animals had free access to all compartments and received a prime dose of MPH (1 mg/kg; i.p.) before testing session.*

For all trials, each subject was captured in its home-cage, injected with its treatment and placed in a waiting-cage similar to its home-cage. It was then recaptured, placed in the transport-cage and taken to the test room where it was released into the antechamber of the CPP box. After the end of each trial, the CPP box was cleaned with 70% alcohol. Animals were tested randomly and sessions were held between 07:30-11:30h.

For all trials, the any maze software (Soelting Co., USA) automatically tracked via the top-view camera the marmosets' total distance and average speed traveled within the CPP box, as well as the time spent in each compartment. In addition, an experienced observer with a 95% intra-rater reliability, manually scored on the same program the following behaviors: Vigilance (i.e. the duration of continuous sweeping upward or downward movements of the head while stationary); Locomotion (i.e. the duration of continuous movement through de CPP box) (1, 2).

Statistical analysis was completed with the SPSS software (Windows Version 23.0; IBM Corporation, NY, USA). Data were analyzed using the paired *t*-test for differences in the locomotor and vigilance behaviors; the time in the MPH-paired and SAL-paired on pre and post-CPP(1, 2). A repeated measures one-way analysis of variance (RM ANOVA) was used to analyze the time in the MPH-paired compartment through all the trials. Subsequent multiple pair-wise comparisons were held with Tukey's test whenever applicable. Significance level for all tests was set at $P< 0.05$.

III. RESULTS

We found that marmosets did habituate to the CPP box, as we found a significant reduction on the locomotion (t_4 =2.92, P = 0.043; Table 1), and no increase in vigilance through the habituation trials (t_4 = -2.99, P = 0.40; Table. 1). Also, subjects did not have an initial preference for either side of the apparatus (t_4 = -0.59, P = 0.5; Fig. 1). After 5 alternate days of MPH, the marmosets spent significantly more time in the MPH-paired compartment in comparison to the SAL-paired at post-CPP (t_4 = -9.96, P = 0.001; Fig. 1) and to the MPH-paired at pre-CPP session (t_4 = -4.826, P = 0.008; Fig. 1). As for the use of CBD on extinction we found a significant

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difference between the trials ($F_{8.32}$ = 4.886, P = 0.031; Fig. 2). According to the pair-wise comparisons, we found a significant difference in time in the MPH-paired zone between pre-CPP x post-CPP (P = 0.008), post-CPP x Extinction 4 (P = 0.016) and Extinction 5 (P = 0.033) and Retest x pre-CPP (P = 0.004).

Table 1. Time marmosets spent (mean ± SEM) in locomotion and vigilance on both habituation trials and first and last conditioning trials.

Parameter	Trial			
	Habituation1	Habituation2	Conditioning1	Conditioning5
Locomotion (s)	69±16	55±16*	43±16	40±6
Vigilance (s)	718±43	747±43	730±69	695±95

*P< 0.05 Habituation 1 vs. Habituation 2

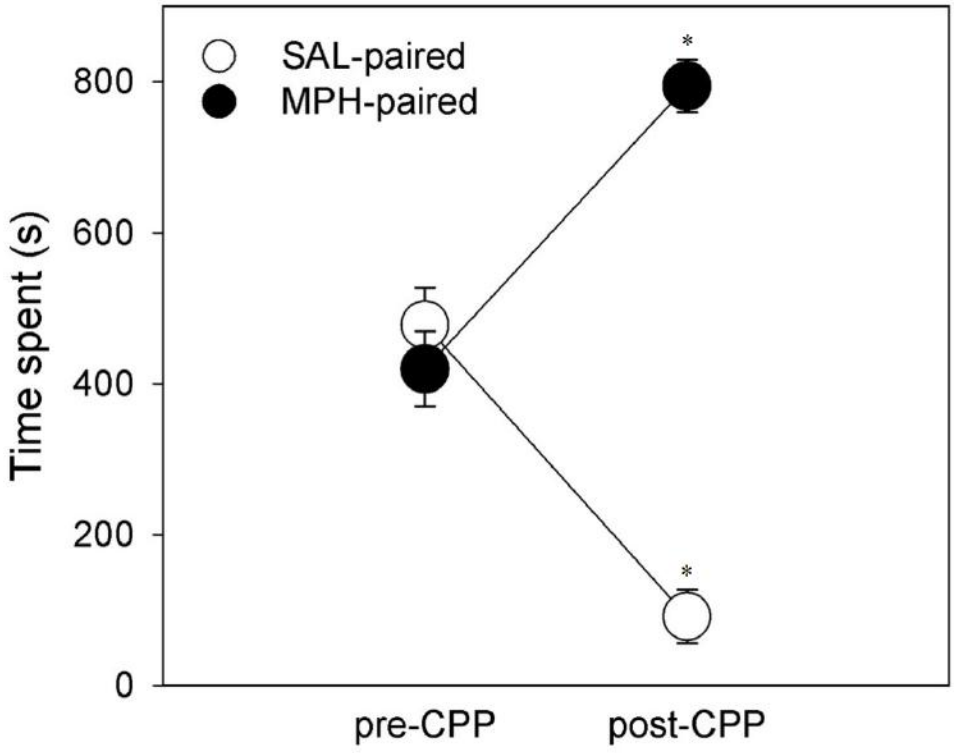

*Fig. 1. Time marmosets (n=5) spent (mean±SEM; in seconds) in the methylphenidate (MPH) paired compartment and the saline (SAL) paired compartment of the CPP box before (pre-CPP; last habituation trial) and after (post-CPP; test trial) the conditioning trials. *P < 0.05 pre-CPP levels.*

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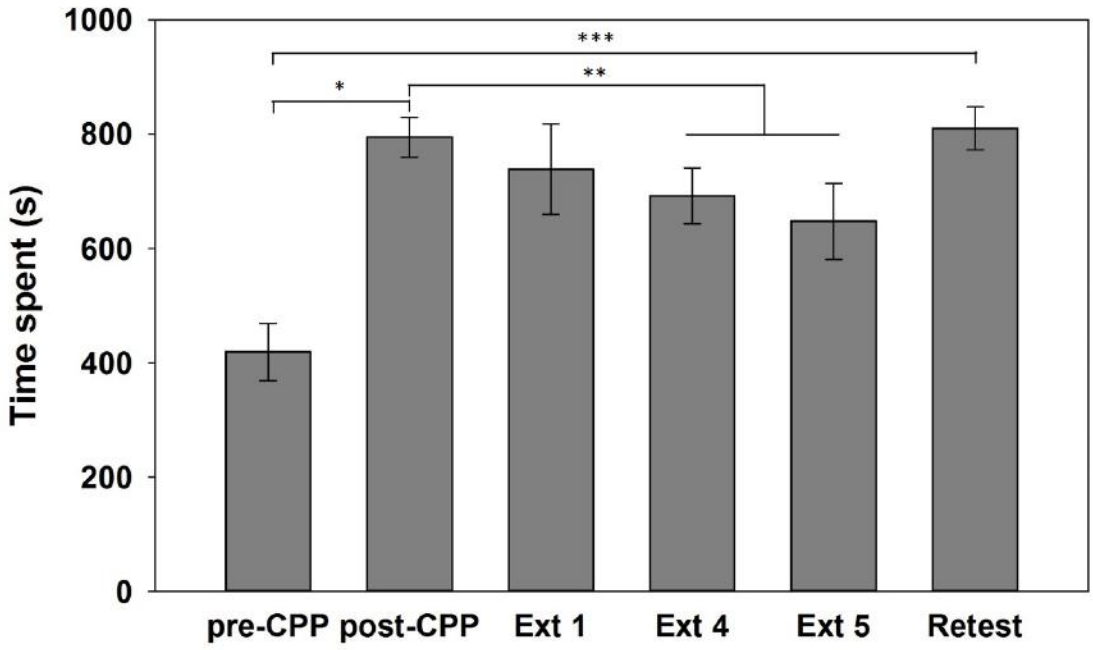

*Fig. 2. Time marmosets (n=5) spent (mean ± SEM; in seconds) in the methylphenidate (MPH) paired compartment of the CPP box before (pre-CPP; last habituation trial) and after (post-CPP; test trial) the conditioning, on the first, fourth e fifth extinction trial (Ext) and on Retest. *P < 0. 05 post-CPP vs. pre-CPP; **P < 0.05 post-CPP vs. Ext4 and Ext5; ***P < 0.05 pre-CPP vs. Retest.*

IV. DISCUSSION

To the best of our knowledge, this paper is the first study focused on the rewarding properties of MPH in NHP using the CPP behavioral paradigm. Results from the present study suggest that MPH has rewarding effect as indicated by the reinforcing effect of MPH-induced CPP in NHP. Our results are in parallel with previous evidence in male rats (16). In this study, the marmosets spent significantly more time in the MPH-paired compartment in comparison to the SAL-paired at post-CPP.

MPH acts as a DA and Norepinephrine (NE) transporter inhibitor, leaving behind high levels of monoamines in the synaptic cleft, which will ultimately increase the level of extracellular dopamine in the brain (17). It is generally accepted that DA action in the Nucleus accumbens mediates the rewarding effects of MPH (18). For example, MPH and cocaine have similar actions at the dopamine transporter (DAT) and produce comparable increases in synaptic dopamine levels in baboons (19).

Though, neurobiological mechanisms underlying the therapeutic effects of MPH in young NHP, particularly marmoset monkey, are not known. One possibility is that the key role of MPH effects involves dopaminergic D1 receptors, mediating the rewarding and reinforcing that produces long-lasting conditioning effects and reinstatement.

Vulnerability to relapse is a chronic condition in drug use disorders (20). Results from our study showed that the CBD administration could affect extinction phase of MPH-induced CPP while did not decrease reinstatement. First we argued that CBD is able to broadly block reward mechanisms as well as affect brain centers that lead to relapse. Animal studies have discovered many beneficial effects of CBD relevant for several relapse-promoting conditions including sensitivity to drug-related contexts and stress, anxiety, and impaired impulse control (21). One study found that daily injections of CBD after conditioning trials but during preference trials diminished preference-seeking behavior in the face of drug-related cues and potentiated the extinction of both amphetamine-induced and cocaine-induced CPP learning. Thus, CBD facilitates the extinction of amphetamine and cocaine addiction and prevents cue-induced relapse (11).

Our findings are in line with previous work showing that CBD (10 and 20 mg/kg, i.p.) did not affect lever pressing induced by heroin during extinction training (13). In another study, CBD does not exhibit an impact on the alcohol addiction intoxication phase in humans, and again, no data were found on the other phases of this addiction (22).

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CBD probably has interaction with dopamine receptors, which play a crucial role in regulating many aspects of behavior and cognition especially reward-seeking. Apart from dopamine, other neurotransmitter systems may be involved in drug reinforcement initiation including serotonin (5-HT), NE, glutamate (GLU), GABA, opioid peptides and endocannabinoids (23). CBD help modulate the endocannabinoid system, it can influence the release of neurotransmitters as well as play a role in the modulation of extracellular levels of DA in the brain (24). In our study it appears that CBD given alone has little effect on CPP. For example, Long-Evans rats treated with 10 mg/kg CBD indicated neither CPP nor CPA (25). It is also important to note that in this initial study, we used only a dose of CBD that is effective during the conditioning and extinction sessions. Therefore, it is possible that lower or higher doses of drugs may have differential effects on factors that facilitate or inhibit the reward systems in NHP.

These results demonstrate that MPH is a reinforcer and that its reinforcing efficacy may be associated with brain's reward circuitry following increased dopamine activity. Daily injection of MPH may have dramatic and longer-term impact on brain and tend to lead to reinstatement. Also these results show that the CBD affect extinction period but could not decrease reinstatement to MPH.

Finally, it is believed that further studies are needed to clarify the real impact of the use of psychostimulants, especially MPH, on the developing of behavioral sensitization and neural mechanisms of relapse. It should be noted that effects of MPH on reinstatement depend on several factors, such as the animal model, drug dose administered, type of experimental parameters and also role of genetic condition as well as sex.

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